Inflammation, not hyperhomocysteinemia, is related to oxidative stress and hemostatic and endothelial dysfunction in uremia

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Inflammation, not hyperhomocysteinemia, is related to oxidative stress and hemostatic and endothelial dysfunction in uremia.

Background. Several cardiovascular risk factors are present in patients with chronic renal failure (CRF), among which are systemic inflammation and hyperhomocysteinemia. Increased oxidative stress, endothelial activation/dysfunction, and coagulation activation are considered integral components of the inflammatory response, but have also been proposed as mediators of plasma homocysteine (tHcy)-induced cell damage. Using correlation analysis, we assessed the relative contributions of inflammation and hyperhomocysteinemia in the abnormal oxidative stress, endothelial activation/dysfunction, and hemostasis activation in patients with CRF.

Methods. The relationships of inflammatory proteins and tHcy with plasma markers of these processes were studied in 64 patients with CRF (serum creatinine = 326 ± 319 μmol/L) on conservative treatment, comparing the results with healthy controls (N = 15 to 40, depending on the measured variable) of similar sex and age.

Results. Patients had significant increases in inflammatory cytokines (TNF-α and IL-8) and acute-phase proteins (C-reactive protein, fibrinogen and α1-antitrypsin). tHcy was increased in 87.5% of patients (mean = 27.1 μmol/L, range 6.5 to 118). Patients had significant increases in (1) indices of oxidative stress: TBARS (thiobarbituric acid-reactive species), a marker of lipid peroxidation and AOPP (advanced oxidation protein products), a marker of protein oxidation; (2) endothelial cell markers such as von Willebrand factor (vWF:Ag), soluble ICAM-1 and soluble thrombomodulin (sTM); (3) markers of intravascular thrombin generation: thrombin-antithrombin complexes (TAT) and prothrombin fragment F1+2 (PF1+2); and (4) indices of activation of fibrinolysis: plasmin-antiplasmin complexes (PAP), fibrin degradation products (FnDP) and fibrinogen degradation products (FgDP). tHcy was significantly correlated with plasma creatinine (r = 0.29, P < 0.018) and with serum folate (r = −0.38, P < 0.002). However, no significant correlations were observed between tHcy and TBARS, AOPP, vWF:Ag, sICAM-1, sTM, TAT, F1+2, sTF, PAP, FnDP, and FgDP. Conversely, acute-phase proteins showed significant, positive correlations with most markers of oxidative stress, endothelial dysfunction and hemostatic activation.

Conclusions. Systemic inflammation, which is closely associated with augmented oxidative stress, endothelial cell dysfunction and hemostatic activation, emerges as a major cardiovascular risk factor in CRF. tHcy is unrelated to these events. Thus, alternative mechanisms through which hyperhomocysteinemia could predispose to vascular lesion and thrombotic events in CRF needs to be investigated.

Ischemic cardiovascular disease associated with accelerated atherosclerosis is the most important cause of death in patients at all stages of progressive kidney disease [1]. Hyperhomocysteinemia, an independent cardiovascular risk marker [2], is present in 85 to 100% of these patients in direct relation to the severity of renal failure [3]; however, its role as a causal cardiovascular risk factor is debated [4]. According to recent prospective studies, the ability of plasma homocysteine (tHcy) to predict cardiovascular events or death in end-stage renal disease is controversial, revealing either a graded increase in relative risk with the rise of tHcy [5] or a worse survival rate in patients with lower tHcy [6].

Mechanisms proposed for the vascular damage induced by hyperhomocysteinemia are heterogeneous, including impaired nitric oxide-dependent vasodilation, endothelial dysfunction and injury, increased oxidative stress, and induction of a procoagulant state [7]. These processes that are involved in the pathophysiology of atherothrombosis also are commonly found in patients...
with chronic renal failure (CRF). In fact, the progression of kidney failure is associated with impaired endothelium-dependent vasodilation [8], elevated plasma markers of endothelial dysfunction [9], enhanced oxidative stress [10, 11], and intravascular indices of hemostatic activation [12, 13]. Whether these alterations in vivo are related to or partially dependent on the elevation of tHcy is currently unknown. On the other hand, such processes are intrinsic constituents of systemic inflammation, a frequent feature in these patients [14, 15] that is a cardiovascular risk factor [16, 17]. In this context, the links between systemic inflammation with in vivo oxidative stress [18] and with endothelial cell [19] and hemostatic activation [20, 21] are well known.

Accordingly, the aim of this study was to measure tHcy and plasma markers of inflammatory response and analyze their relationships with coexisting endothelial dysfunction, oxidative stress, and hemostatic activation in patients with different degrees of CRF under conservative treatment.

**METHODS**

**Patients**

Sixty-four patients were enrolled in this study before initiating chronic maintenance hemodialysis or peritoneal dialysis. Demographic data of the patients are presented in Table 1. This population had no nephrotic syndrome, diabetes mellitus, systemic vasculitis or lupus erythematosus, infections, or other diseases known to be associated with clinical or laboratory perturbations of hemostasis. The cause of chronic renal insufficiency was hypertensive renal disease (2 patients), chronic glomerulopathies (15 patients), tubulointerstitial disease (3 patients), autosomal-dominant polycystic kidney disease (3 patients), other nephropathies (2 patients), and unknown etiology at the time of the study (39 patients). Blood pressure at the time of study was less than 180/100 mm Hg with the patient in a supine position. Patients did not receive anticoagulants or antiplatelet drugs and had not been transfused with blood products during the preceding 30 days. They did not take vitamin supplements containing folic acid or vitamins B12, B6, or vitamin C from at least two months before the study. Forty healthy subjects (mean age of 49 ± 13 years, 20 women and 20 men) were studied in parallel with patients. Samples from all these individuals served as normal controls for tHcy. As detailed in the Results section, for all other variables, random samples from at least 15 of these 40 healthy individuals served as controls. Patients gave written consent to participate in the study, which was approved by the medical ethics committee of our institution.

**Blood sampling and processing**

Blood was collected from a forearm vein using a 19-gauge butterfly needle after the patient had fasted for 10 to 12 hours. Two milliliters were used for measurements of serum creatinine, blood urea nitrogen (BUN), albumin, total cholesterol, folate and vitamin B12; an additional 2 mL were drawn in ethylenediaminetetraacetic acid (EDTA). Aliquots were used to measure hematocrit and blood platelet count, and the platelet-poor plasma was frozen at −70°C within 30 minutes after blood collection and saved for tHcy determination. Nine milliliters were drawn in two vacuum tubes, each containing 0.5 mL of sodium citrate (0.13 mol/L, 9/1 vol/vol), which were immediately placed in melting ice for 15 minutes, centrifuged for 15 minutes at 3200 × g at 4°C, and the collected platelet-poor plasma aliquoted and stored at −70°C until processed. These samples were used for measurements of inflammatory cytokines, acute-phase proteins, and plasma markers of endothelial dysfunction/activation, of oxidative stress and of hemostasis activation.

**Laboratory tests**

Plasma creatinine was measured by kinetic Jaffé method (Autonalyzer Hitachi 917; Roche). Creatinine clearance was calculated using the Cockcroft-Gault formula [22]. Serum albumin was measured with bromocresol green method (Autoanalyzer Hitachi 917; Roche). Fasting tHcy in EDTA plasma was measured by high pressure liquid chromatography (HPLC) with fluorometric detection (Chromsystems, München, Germany). Folate and vitamin B12 in serum were determined by ion capture and microparticle immunoassay, respectively (IMX® Folate and AxSYM B12; Abbott Laboratory, Abbott Park, IL, USA).

Markers of systemic inflammatory reaction included plasma levels of inflammatory cytokines and acute-phase proteins: tumor necrosis factor-α (TNFα; Croma- genix, Sweden), by enzyme-linked immunosorbent assay (ELISA), interleukin-8 (IL-8; R&D Systems, Minneapolis, MN, USA) by ELISA, C-reactive protein (CRP) by competitive immunoassay with a sensitivity of 0.18 mg/L [23, 24]; plasma fibrinogen by the Clauss technique as
controls was performed by the unpaired Student t coefficient, for data fitting or not fitting a normal distribution index between tHcy and serum vitamin B12 (mean 359, range 126 to 1475 pmol/L). Vitamin B12 level in patients (mean 36) was slightly below the lower limit of the normal range according to their sex (women, 7.7 ± 1.6 μmol/L; men, 9.2 ± 2.6 μmol/L). The mean serum folate concentration in patients with CRF (11.7 ± 5.9 nmol/L) was slightly below the lower limit of the normal range of our laboratory (12 to 33 nmol/L). Vitamin B12 level in patients (mean 359, range 126 to 1475 pmol/L) was within the normal range (164 to 835 pmol/L). tHcy was positively correlated with plasma creatinine (r = 0.29, p = 0.018) and negatively correlated with serum folate level (r = −0.38, p = 0.002). The correlation index between tHcy and serum vitamin B12 (r = −0.18) was not statistically significant. Plasma levels of inflammatory cytokines and acute phase proteins were significantly higher in patients than in controls.

Similarly, patients had increased plasma markers of oxidative stress, endothelial dysfunction and hemostatic activation, as shown in Table 3. Additionally, a matrix of significant, positive associations among these indices was observed. Plasma concentration of TAT complexes was significantly correlated with other markers of hemostatic activation, such as F1+2 (P = 0.003), PAP (P = 0.003), FnDP and FgDP (P < 0.0001). TAT also was correlated with TBARS, a marker of lipid peroxidation (P = 0.008), and with plasma vWF Ag, a marker of endothelial activation/dysfunction (P = 0.006). TBARS and AOPP, an index of protein oxidation, were significantly related to markers of endothelial dysfunction and hemostatic activation, that is, TBARS was positively correlated with FnDP and FgDP (P = 0.008 and 0.004) and with sTM (P = 0.02). Finally, significant correlations between AOPP with PAP complexes (P = 0.01) and vWF Ag (P = 0.003) were observed.

Markers of oxidative stress, endothelial dysfunction, and hemostatic activation were correlated with plasma creatinine, tHcy and acute phase proteins (Table 4). As shown, plasma creatinine was statistically correlated with

### Table 2. Plasma concentrations of total homocysteine, inflammatory cytokines and acute-phase proteins in patients with chronic renal failure and healthy controls

<table>
<thead>
<tr>
<th>Plasma concentration†</th>
<th>Patients (N = 64)</th>
<th>Controls (N = 16)</th>
<th>P⁷</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total homocysteine μmol/L</td>
<td>27.1 (6.5–118)</td>
<td>8.4 (5.0–18) (N = 40)</td>
<td>0.0001</td>
</tr>
<tr>
<td>TNF-α pg/mL</td>
<td>36.4 (3.8–137)</td>
<td>19.9 (2.4–43) (N = 16)</td>
<td>0.001</td>
</tr>
<tr>
<td>IL-6 ng/mL</td>
<td>12.9 (0.07–106)</td>
<td>3.2 (0.23–14.6) (N = 24)</td>
<td>0.0014</td>
</tr>
<tr>
<td>C-reactive protein mg/L</td>
<td>45 (32–283)</td>
<td>3.6 (1.6–16) (N = 36)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Fibrinogen g/L</td>
<td>4.3 ± 1.4</td>
<td>2.2 ± 4.7 (N = 16)</td>
<td>0.0001</td>
</tr>
<tr>
<td>α1-antitrypsin mg/dL</td>
<td>143 ± 33</td>
<td>110 ± 20 (N = 16)</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

† Values represent mean ± 1 SD for variables with normal distribution and mean and range for data not fitting a normal distribution

⁷ Non-paired t test or Mann-Whitney U test

### RESULTS

Table 2 shows the plasma concentrations of tHcy, inflammatory cytokines, and acute phase proteins in patients and controls. Plasma tHcy was significantly higher in uremic subjects. Only 8 out of 64 patients (12.5%) had tHcy values within the normal range according to their sex (women, 7.7 ± 1.6 μmol/L; men, 9.2 ± 2.6 μmol/L).
several markers of endothelial dysfunction, oxidative stress and hemostatic activation, denoting their dependence on the severity of CRF. Inflammatory proteins were significantly correlated with most markers of these processes. Conversely, tHcy was not significantly correlated with any of them. Figure 1 illustrates this difference, depicting the association of fibrinogen and tHcy with selected markers of endothelial dysfunction, oxidative damage and hemostasis activation.

No statistical relationship between tHcy and inflammatory proteins was detected (data not shown).

**DISCUSSION**

This study essentially shows that indices of lipid peroxidation, protein oxidative damage, endothelium activation/dysfunction, and coagulation/fibrinolysis activation in patients with predialysis CRF are related to systemic inflammation, but not to hyperhomocysteinemia. Almost 90% of the patients with CRF had increased tHcy, which was significantly related to the severity of kidney insufficiency and to serum folate levels. Even though a recent article associated hyperhomocysteinemia with vascular inflammation in a murine model [27], no correlation was found between tHcy and plasma inflammatory markers, suggesting that both are independent processes. Conversely, tHcy was not significantly correlated with any of them. Figure 1 illustrates this difference, depicting the association of fibrinogen and tHcy with selected markers of endothelial dysfunction, oxidative damage and hemostasis activation.

No statistical relationship between tHcy and inflammatory proteins was detected (data not shown).

**Table 3.** Plasma markers of oxidative stress, endothelial activation/dysfunction and hemostatic activation in patients with chronic renal failure and healthy controls

<table>
<thead>
<tr>
<th>Plasma concentration</th>
<th>Patients (N = 64)</th>
<th>Controls</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBARS μmol/L</td>
<td>1.98 ± 0.48</td>
<td>1.55 ± 0.39 (N = 15)</td>
<td>0.009</td>
</tr>
<tr>
<td>AOPP mmol, eq. chloramine T</td>
<td>281 (45–915)</td>
<td>121 (14–414) (N = 25)</td>
<td>0.0001</td>
</tr>
<tr>
<td>von Willebrand factor %</td>
<td>182 ± 78</td>
<td>116 ± 46 (N = 40)</td>
<td>0.001</td>
</tr>
<tr>
<td>Soluble thrombomodulin ng/mL</td>
<td>15.7 ± 2.1</td>
<td>5.7 ± 0.45 (N = 16)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Soluble ICAM-1 ng/mL</td>
<td>301 (174–508)</td>
<td>233 (179–275) (N = 15)</td>
<td>0.0001</td>
</tr>
<tr>
<td>TAT μg/L</td>
<td>3.3 (0.94–14.2)</td>
<td>2.1 (0.84–4.7) (N = 30)</td>
<td>0.03</td>
</tr>
<tr>
<td>PAPF1.2 μmol/L</td>
<td>3.0 ± 1.1</td>
<td>1.8 ± 0.8 (N = 25)</td>
<td>0.0001</td>
</tr>
<tr>
<td>PAP μg/L</td>
<td>874 (146–2302)</td>
<td>475 (321–805) (N = 16)</td>
<td>0.0001</td>
</tr>
<tr>
<td>FnDP ngF/ml</td>
<td>675 (118–3622)</td>
<td>173 (9–545) (N = 23)</td>
<td>0.0001</td>
</tr>
<tr>
<td>FgDP mgF/ml</td>
<td>391 (20–5875)</td>
<td>89 (20–236) (N = 27)</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

Abbreviations are: TRARS, thioarbituric acid-reactive species; AOPP, advanced oxidation protein products; TAT, thrombin-antithrombin complex; PAPF1.2, prothrombin fragment F1.2; PAP, plasmin-antiplasmin complex; FnDP, fibrin degradation products; FgDP, fibrinogen degradation products. Values represent mean ± 1 SD for variables with normal distribution and mean and range for data not fitting a normal distribution. The unpaired Student t test or Mann-Whitney test was used for analyses.

**Table 4.** Correlations of plasma creatinine, total plasma homocysteine (tHcy) and acute-phase proteins with markers of oxidative stress, endothelial activation/dysfunction, and hemostatic activation in patients with chronic renal failure

<table>
<thead>
<tr>
<th>Variable</th>
<th>Plasma creatinine</th>
<th>tHcy</th>
<th>C-reactive protein</th>
<th>Fibrinogen</th>
<th>α1-antitrypsin</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBARS</td>
<td>r = 0.33</td>
<td>r = 0.06</td>
<td>r = 0.21</td>
<td>r = 0.32</td>
<td>r = 0.37</td>
</tr>
<tr>
<td>AOPP</td>
<td>r = 0.01</td>
<td>P = NS</td>
<td>P = NS</td>
<td>P = 0.01</td>
<td>P = 0.005</td>
</tr>
<tr>
<td>vWF</td>
<td>r = 0.29</td>
<td>r = 0.12</td>
<td>r = 0.28</td>
<td>r = 0.46</td>
<td>P = 0.11</td>
</tr>
<tr>
<td>sTM</td>
<td>r = 0.79</td>
<td>r = 0.04</td>
<td>r = 0.02</td>
<td>P = NS</td>
<td>r = 0.0003</td>
</tr>
<tr>
<td>sICAM-1</td>
<td>r = 0.08</td>
<td>P = 0.0001</td>
<td>P = 0.0002</td>
<td>P = 0.0001</td>
<td>P = 0.004</td>
</tr>
<tr>
<td>TAT</td>
<td>r = 0.21</td>
<td>r = 0.08</td>
<td>r = 0.33</td>
<td>r = 0.56</td>
<td>r = 0.35</td>
</tr>
<tr>
<td>PAP</td>
<td>r = 0.21</td>
<td>P = NS</td>
<td>P = NS</td>
<td>P = NS</td>
<td>P = 0.0001</td>
</tr>
<tr>
<td>FnDP</td>
<td>r = 0.32</td>
<td>P = 0.01</td>
<td>P = 0.01</td>
<td>P = NS</td>
<td>P = 0.0001</td>
</tr>
<tr>
<td>FgDP</td>
<td>P = 0.001</td>
<td>P = NS</td>
<td>P = 0.014</td>
<td>P = 0.0001</td>
<td>P = 0.004</td>
</tr>
</tbody>
</table>

Pearson’s correlation or Spearman rank correlation were used for data fitting or not fitting a normal distribution, respectively. Abbreviations are in the footnote of Table 3.
Activating reactive oxygen species and, furthermore, may potentiate peroxyl radical-mediated nitric oxide inactivation [29], with resultant endothelial dysfunction and injury. Homocysteine-induced endothelium damage also may be explained by several other mechanisms, as shown in the in vitro systems, and these account for the transformation of its normal antithrombotic phenotype into a prothrombotic one [7]. Thrombin generation may be facilitated through the tHcy-induced monocyte expression of tissue factor, an intravascular initiator of coagulation, by a mechanism independent of oxidative stress [30]. Taken together, these observations strongly suggest that the enhanced oxidative stress, endothelial dysfunction and hemostatic activation that are regularly observed in uremic patients could have a close relationship with the elevated tHcy concentration in these patients. However, as discussed later in this article, the results of our study do not support this hypothesis.

Abnormally high plasma indices of lipid peroxidation (TBARS) and oxidative protein damage (AOPP) in patients with predialysis CRF confirmed previous observations [10, 11]. Even though the TBARS colorimetric reaction used in our study is less sensitive and specific than HPLC-based methods [11], elevated plasma levels of malondialdehyde in patients with CRF have been found with both techniques. We did not detect any relationship between these protein and lipid oxidative markers and tHcy. Accordingly, our study in plasma samples of patients with CRF does not support the concept that hyperhomocysteinemia toxicity is mediated through a mechanism involving oxidative damage [28]. In this context, our results are more in line with recent reports disputing the notion that an enhanced tHcy-induced oxidant stress is a major contributor to atherothrombotic complications [31, 32].

While significant increases were found in plasma levels of von Willebrand factor, sTM and sICAM-1, as markers of endothelium activation/dysfunction, no statistical relationship with tHcy was detected. Our study did not assess the relationship between tHcy and endothelium-dependent vasodilation, a topic raised by the observation that monkeys with diet-induced moderate hyperhomocysteinemia showed an impaired response to the endothelium-dependent vasodilators acetylcholine and adeno-
Patients with CRF show features compatible with chronic, systemic inflammatory response [14, 15]. The increased plasma levels of inflammatory cytokines and acute phase proteins observed in predialysis patients confirm these observations and indicate that artificial surfaces or dialytic procedures are not causally involved in the inflammatory reaction. The significant interrelationships between inflammatory proteins and the majority of markers of oxidative damage, endothelial dysfunction, and hemostatic activation suggest that the systemic inflammation of uremia is a common and unifying factor underlying these processes. The observed associations can be explained on the basis that activation of inflammatory cells generates reactive oxygen species and inflammation is intimately associated with activation of both endothelium [19] and hemostatic system [20]. In CRF, the inflammatory response coexists with increased oxidative stress [18, 41] and endothelial dysfunction [34, 42, 43]. Also, activation of hemostasis is a common feature of uremia [12, 25, 38, 44], and its link with inflammation in this disease also has been reported [21, 45]. In this context, CRF can be viewed as a clinical model of mild, protracted systemic inflammation that is intrinsically associated with oxidative stress, endothelial dysfunction and hemostatic activation, which are all important mediators of atherosclerosis and thrombotic events.

Many studies show that the association between tHcy and cardiovascular disease is strong, although firm evidence for a causal relationship is still lacking [46]. Our study did not address the role of increased tHcy as a cardiovascular risk factor in CRF, and we cannot discard this possibility. However, our data conflict with the prevailing notion that homocysteine-mediated damage in CRF results from increased oxidative damage, endothelial dysfunction, coagulation activation or inhibition of the fibrinolytic process. Alternative mechanisms through which elevation of tHcy predisposes to vascular lesions and thrombotic events in CRF should be investigated. Noteworthy, these mediators of cardiovascular disease are associated with the inflammatory response, whose initial triggering event is still unknown. In this context, our observations add evidence to the theory that chronic inflammation is a major determinant of the atherothrombotic risk of patients with CRF.
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